NEWLY PHOSPHORYLATED PROTEINS ASSOCIATED WITH CYTOPLASMIC dRNA

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1. Introduction

It has recently been shown that ribosomes from different eukaryotic cells contain phosphorylated proteins [1–3] which can in part be removed by common ribosome-dissociating agents [3]. The number of these proteins has not been clearly defined. Using disc electrophoresis, various numbers of proteins have been reported [1, 2, 4]. Evidence has been presented that in rat liver the phosphorylating process can be markedly increased *in vivo* by administration of glucagon [4]. It can also take place on ribosomes *in vitro* by a cAMP-dependent protein kinase reaction [3].

The present investigation demonstrates that the major part of the newly phosphorylated protein located in the "ribosomal" fraction is bound to the dRNA† –protein complex from the cytoplasm, that is, the fraction related to the "informosomes" of Spirin [5].

2. Methods

Solid plasmocyte tumors RPC_5, transplanted on Balb/c mice, were used. Tritiated uridine or ^{32}P-orthophosphate has been injected in tumor bearing mice as described in the legend of fig. 2. By means of a Potter-Elvehjem tissue grinder excised tumors were homogenized in the cold with 5 vol.of a buffer consisting of: 0.020 M Tris-HCl pH 7.6; 0.050 M KCl; Mg acetate 0.25 M sucrose. The homogenate was centrifuged 15 min at 20,000 *g* and a particulate fraction was then obtained from the supernatant by centrifugation at 200,000 *g* for 2 hr. The pellet (fraction F200, fig. 2) obtained from approx. 2 *g* wet weight of tumor, was resuspended in 10 ml of the same buffer made either 1% with respect to deoxycholate or 0.5% with Triton X-100, and 6 ml of this suspension was spread on top of a double layer of dense sucrose as described in fig. 2. Centrifugation was carried out in a Spinco rotor R65 during 14 hr at 45,000 rpm at 1°. The measured volumes of supernatant indicated in fig. 2 were pipetted off and the pellet was resuspended in 1 ml of buffer.

Isopycnic sedimentation in CsCl density gradients was performed on formaldehyde fixed material as described earlier [6].

Radioactivity was measured by the method of Mans and Novelli [7] adapted to our purposes: thus, the filter paper strips carrying the radioactive material were processed as follows. Paper strips were first treated by 10% TCA at 0° in order to extract acid soluble compounds. Phospholipids were then extracted by chloroform–methanol (2:1, v/v). RNA was then hydrolyzed by 5% TCA at 95°. The residual ^{32}P will essentially all be contained in phosphoprotein.

Phosphoserine and phosphothreonine were identified in fractions 1 E and 2 B (fig. 2). These fractions

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† Abbreviations used: rRNA: ribosomal RNA; dRNA: DNA-like RNA; mlRNA: messenger-like RNA; DOC: sodium deoxycholate; TCA: trichloracetic acid.

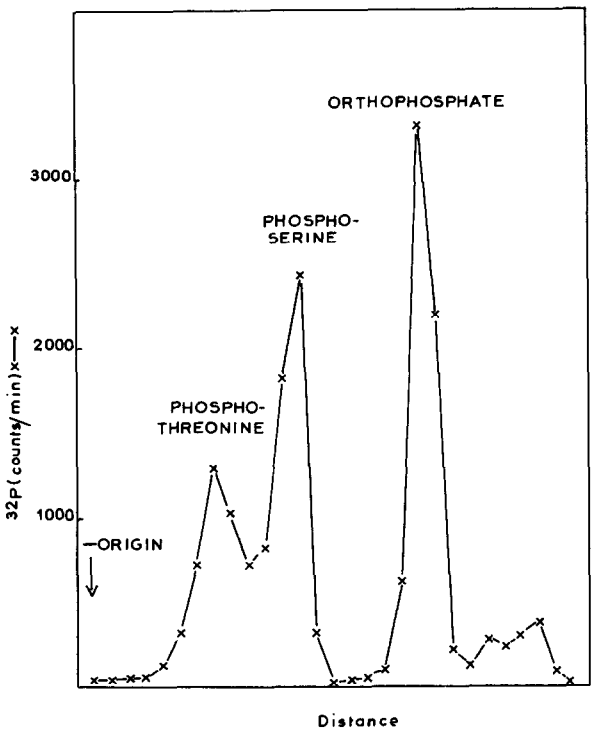

Fig. 1. Identification of ^{32}P-labelled phosphoserine and phosphothreonine obtained by hydrochloric acid hydrolysis from fraction 2B (see fig. 2), and separated by paper electrophoresis. The positions of authentic, co-electrophoresed phosphoserine and phosphothreonine localized by ninhydrin spray, are indicated by arrows.

were precipitated and washed in centrifuge tubes with 0.6 N perchloric acid, lipid extracted and then hydrolyzed by 2 N HCl at 100° during 10 hr. The hydrolysate, lyophilized and redissolved in a water–acetone mixture, was co-electrophoresed on paper with cold phosphoserine and phosphothreonine during 2 hr at 2,000 V. Radioactivity was determined by scintillation counting on strips cut from the paper pherogram.

3. Results and discussion

3.1. *Radioactive labelling of compounds*

In plasmocyte tumors from the mouse, it is possible, following even high doses of actinomycin D, to selectively label dRNA during several hours [8]. Previous to the antibiotic administration rRNA can be long-term labelled by a different radioactive marker.

The ^{32}P-orthophosphate, chosen for labelling under actinomycin D, is incorporated not only into dRNA, but also into the acid-insoluble phospholipids of lipoproteins, and into phosphoproteins. By selective extraction followed by hydrolysis of the cytoplasmic fraction, either on paper strips, or on the acid precipitate, the radioactivity which can be ascribed to each of these compounds can be distinguished.

3.2. *Identification of phosphoprotein*

In the hot TCA insoluble residue, the ^{32}P label was made totally acid soluble by 0.5 N KOH at 37° during 15 hr while pronase at 50 μg/ml solubilized 80% of it in 15 hr at 37°. The products of hydrochloric acid hydrolysis of the TCA insoluble residue, analyzed by high voltage electrophoresis, contained large amounts of ^{32}P-phosphoserine and ^{32}P-phosphothreonine (fig. 1).

3.3. *Identification of cytoplasmic dRNA-protein particles containing phosphoprotein.*

Cytoplasmic extracts sedimentable between 20,000 *g* and 200,000 *g* either treated by Triton X-100 (series 1) or by DOC (series 2) were fractionated on a discontinuous sucrose-density gradient as indicated in fig. 2. This figure gives the pattern of repartition of the three major ^{32}P-labelled compounds along this gradient. It can be seen that the radioactivities of dRNA and of phosphoprotein are in a fairly constant proportion to each other, but that this relation does not exist between phosphoprotein and rRNA or phosphoprotein and phospholipid. Thus, in the polysome fraction (1E from fig. 2) obtained after treatment by Triton X-100, dRNA and phosphoprotein exist in quite a high amount. Yet, in the deoxycholate treated fraction, dRNA and phosphoprotein are mainly contained in a low density zone with low ribosome content (2B from fig. 2). A possible linkage between phosphoproteins and dRNA appears further substantiated by the data from isopycnic sedimentation of the F200 fraction in a CsCl density gradient (fig. 3).

The phosphoprotein and dRNA containing particles have a buoyant density in CsCl of nearly 1.4, which is a density characteristic of informosome-like particles, while only small amounts of phosphoprotein are detected in the ribosomal peak of buoyant density 1.56.

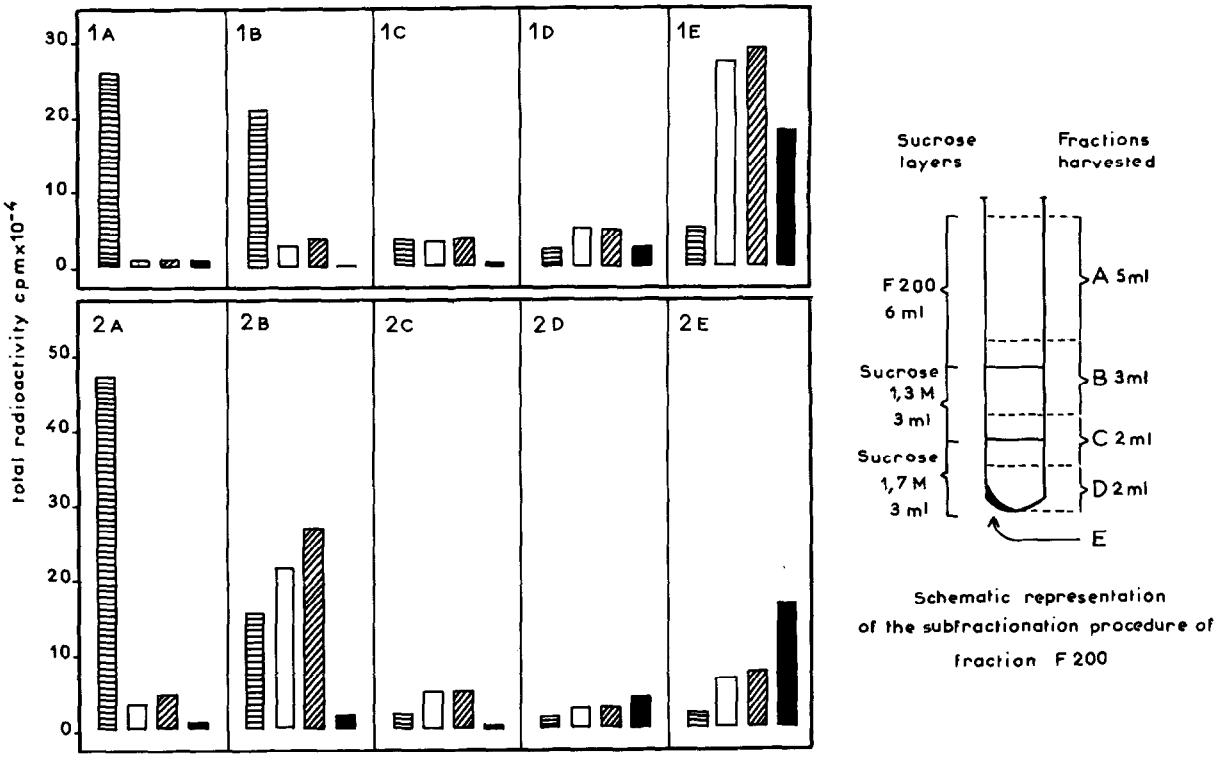

Fig. 2. Distribution of ^{32}P-labelled, acid insoluble compounds of fraction F200, after centrifugation in a discontinuous sucrose density gradient, built up as illustrated at the right of the figure. The length of each bar represents the total radioactivity of each compound in each fraction taken from the gradient after centrifugation. Tumor-bearing mice were injected intraperitoneally, first with 0.1 mCi tritiated uridine; 20 hr later, they received 100 μg of Actinomycin D and, after 30 min, 2 mCi of ^{32}P-orthophosphate. Mice were killed 3.5 hr after injection of ^{32}P-orthophosphate. Series 1 is extraction of pellet F200 with Triton X-100; series 2 with DOC.

▤ : ^{32}P radioactivity from phospholipids.
□ : ^{32}P radioactivity from dRNA.
▨ : ^{32}P radioactivity from phosphoproteins.
■ : ^{3}H radioactivity from long-term labelled rRNA.

In agreement with many papers dealing with different eukaryotic cells [5] we have found in the case of mouse plasmocytoma, that cytoplasmic informosomes have sedimentation coefficients in sucrose density gradients which range from 40 S to 90 S [9]. Thus, even after dissociation by EDTA, informosomes co-sediment with ribosomal particles and are very difficult to separate from them. It is therefore possible that the ribosomal phosphoproteins described by others are contaminated by informosomal phosphoproteins. In our material only a small fraction of the total ^{32}P-phosphoproteins migrate with the ribosomes and it may be that these are in fact polysome associated informosomes. One cannot conclude from the present data whether true ribosomal phosphoproteins exist.

An interesting problem would be to investigate whether phosphorylation is related to the function of informosomes in protein synthesis. Many authors have stated that one kind of regulation of protein synthesis may take place at the posttranscriptional level [11–13]. Phosphorylation of proteins may perhaps play a role in this regulation. We know furthermore that phosphorylation can be mediated by cyclic AMP under hormonal control [14, 15, 4]. Hence we plan to identify the informosomal phosphoproteins and to study their function in relation to the function of mlRNA.

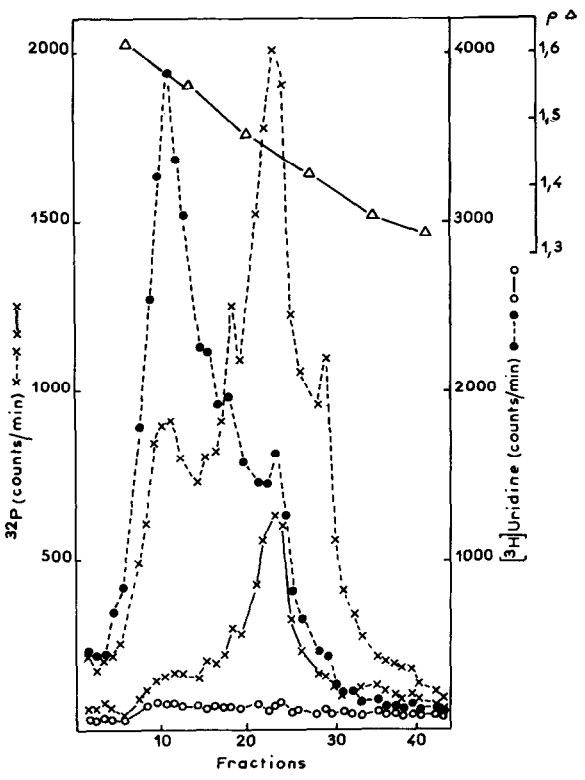

Fig. 3. Isopycnic sedimentation of total formaldehyde-fixed fraction F200 in a preformed CsCl density gradient, centrifuged 16 hr at 45,000 rpm at 10° in a Sw 65 Ti rotor.
(x---x---x): ^{32}P-radioactivity after cold TCA wash.
(x–x–x): ^{32}P-radioactivity after treatment of the same samples with TCA at 95° during 15 min.
(•–•–•): ^{3}H-radioactivity of long-term labeled rRNA after cold TCA wash.
(o–o–o): ^{3}H-radioactivity of samples treated by TCA at 95° during 15 min.

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